Prokaryotic cell membrane-based protein technologies (Review)

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Abstract. The biological cell membranes play a crucial role in living tissues through a heterogeneity of metabolic processes, such as apoptosis, necrosis, autophagy, cell signalling processes and metabolic disorders. Due to the sensitive nature of cell membranes and proteins, the utilization of specialized instruments for carrying out biological experiments such as extraction, isolation, gene expression and protein expression analyses is required. Currently, there are several instruments available for performing biological experiments. The present study selectively discusses the protein bioanalytical techniques, including electrophoresis techniques, chromatographic techniques, sequencing approaches and computational approaches that have been created as a result of difficulties in researching membrane protein and glycoproteomic techniques. Since these techniques have provided an efficient strategy with which to enrich and characterize membrane and plasma-membrane proteomes, some of the recent advancements are also discussed. In addition, the present study focuses particularly on prokaryotic organisms for a better understanding of cell membrane protein technologies. Hopefully, a summary of these studies will help future researchers to understand the gaps behind the technique and its advances.

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1. Introduction

One of the most fundamental parts of a functioning cell is the cellular membrane, where proteins closely related to the membrane mediate important functions, such as selective material mobility and information transmission between the cell and its surroundings (1). Of note >75% of the total membrane proteins are present in the mitochondria and the remaining 25% are present in myelin membranes (2). Membrane proteins can be broadly classified into two types, extrinsic and intrinsic, based on their function and are also classified based on their position attached to the lipid membrane including integral, lipid anchored and peripheral (3). Integral proteins are intrinsic and have a cytoplasmic and extracellular domain; they are transmembrane proteins found in an embedded form with membrane-spanning domains, such as α-helices and multiple β-strands (4). The integral proteins can be otherwise termed as single-pass transmembrane and multi-pass transmembrane proteins based on the polypeptide chain crossing the lipid bilayer (5). Bacteriorhodopsin (single-pass transmembrane protein) and aquaporins (multi-pass transmembrane proteins) are examples of integral proteins. Mostly they are glycosylated and located in the extracellular space, and they are involved in endocytic and secretory pathways (6,7). Peripheral membrane proteins are extrinsic and have hydrophilic domains that transduce intracellular signalling. G protein-coupled receptor (GPCR), receptor tyrosine kinases, protein channels and transporters are some examples of peripheral membrane proteins, and these are expressed in downstream signalling pathways and are involved in cellular changes (8,9). Lipid-anchored proteins are water soluble proteins attached covalently and function either on one side of the cytoplasmic phase or extracellular phase (10). One group of proteins are anchored to the membrane by fatty acyl chain, which are covalently linked to the N-terminal of glycine residue (acylation), a second group of proteins are anchored to the membrane by a hydrocarbon chain attached to the cysteine residue in the C terminus (prenylation), and a third group (glycophosphatidylinositol

anchored proteins) bind to the cell surface and are specialized proteins in the exoplasmic phase of membranes (11).

When proteins are synthesized by pre-existing membranes, they are inserted into the plasma membrane asymmetrically and are distributed across the lipid bilayer. An inside and outside asymmetry distribution of lipid bilayer occurs by the external location of carbohydrates (oligosaccharide) attached to the membrane proteins (12). Oligosaccharide chains are linked via the H-bonds with the glycolipids attached on the extracellular surface. Phospholipid-binding proteins recognize specific phospholipids and transfer them from the plasma membrane to mitochondria and peroxisomes (13). The present review discusses the topics behind protein studies step by step in an aim to provide an understanding of strategies used to perform experiments based on the type of proteins and how they can be processed.

2. Protein separation and purification

Protein molecules can be separated based on the charge, affinity, solubility, size and adsorption properties. Electrophoresis and chromatography principles are the most popular separation technique for protein molecules and some examples of this include ion-exchange chromatography, affinity chromatography, dialysis, ultrafiltration, size-exclusion chromatography (14) and electrophoresis [including capillary electrophoresis, isoelectric focusing and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)] utilized to isolate protein samples (15).

During the biological experimentation process to separate and purify protein samples, several detergent solutions are used. The detergents utilized for protein separation can be categorized into denaturing and non-denaturing detergents. Denaturing detergents are polar molecules that contain charges (anionic or cationic); one such widely used anionic detergent is SDS and cationic detergents include cetyltrimethylammonium bromide. Similarly, non-denaturing detergents were also utilized and are classified based on the material and components used; these include non-ionic detergents, zwitterionic detergents and bile salts. Some popular non-denaturing detergents include Triton X-100, CHAPS and cholate (16).

In a recent study, Zhou *et al* (17) established with a protocol for isolating mitochondrial proteins. They utilized cultured 293T cells for demonstration in which the mitochondrial fraction was isolated from cultured 293T cells in the initial phase and membrane proteins were localized by two methods, sonication and the sodium carbonate method. The sodium carbonate method is utilized to extract integral proteins and the sonication method is used to extract both integral and peripheral proteins from the soluble protein. By contrast, the non-ionizing detergent (Triton-X) and proteolytic enzyme (proteinase K) are used to separate the outer membrane proteins (17).

3. Protein identification and mapping

Protein appears in a 3-dimensional structure and to determine its structure, mapping is performed to identify hot-spot interactions and various other potential functions to recognize functional binding sites and their roles. This can be performed experimentally using various computer aided platforms, as well bioinstrumentation. One such extraordinary analytical technique invention for the identification of unknown proteins is matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MS). It functions on the principle of peptide mass fingerprinting (PMF) in which the unidentified protein molecules are cleaved into small peptides and absolute masses are accurately measured. Finally, the NIST mass spectral libraries is utilized with the PMF data for the identification of anonymous proteins (18). This technique can be applied to various fields, such as microbiology, biotechnology, food chemistry and environmental sciences to uncover the hidden protein from the large population (19).

Nuclear magnetic resonance (NMR) spectroscopy is another technique widely used to obtain structural information of protein, nucleic acids and other biomolecules. It is performed in a very lower energy frequency (radio frequency region of the electromagnetic spectrum) and is based on nuclear spin interactions with a magnetic field; the structure of a molecule is predicted. It is a technique widely used by chemists to predict a chemical structure of an unknown molecule. Chemical shifts and J-couplings (internal nuclear spin interactions) are two key parameters exclusively used by chemists to predict a chemical structure. Processing samples in NMR should be diluted with deuterated solvent, such as chloroform, deuterium oxide and other solvents based on the nature of the material. Acetone, methanol, trichloromethyl, nitrogen gas and a vacuum are some of the substances used to cleanse the NMR tubes. This technique is widely applied in pharmaceutical sciences, metabolomics research and food chemistry (20-22). In addition, reversed-phase-high pressurized liquid chromatography (RP-HPLC) has been utilized in anti-platelet drug determination (ticagrelor) in human blood plasma and in the determination of the dosage form of chemical compounds in pharmaceuticals (23,24). By contrast, for the diagnostic and monitoring parameters in healthcare, enzyme-linked immunosorbent assay is utilized as a significant approach to measure certain types of biomarkers responsible for the cause of disease (25).

Additionally, some of the recent advancements in protein identification include the identification of phosphoprotein and signatures (cyclin D1, ERa and AR S650) using laser capture microdissection-reverse phase protein microarrays by Gallagher *et al* (26).

4. Evolution of sequencing approaches and proteomic studies

The evolution of determining the protein sequence began in the early 1950s with the sequencing of insulin using the Edman degradation method. Edman degradation is the first used method to sequence the amino acids in a peptide and is performed without breaking the peptide bonds between other amino acid residues, amino-terminal residue is labelled and separated from the peptide. This method is also known as N-terminal sequencing, and is utilized to identify unknown proteins, and the quality and identity of proteins can also be determined. The main advantage of this method is that it does not damage the protein entity and a disadvantage is that it is not able to identify multiple proteins simultaneously. Advancements to the Edman method led to the use

of nucleic acids and enzymes to sequence the protein, and sequencing known as the Sanger method. The application of the Edman method is widely applied to product development in chemical and pharmaceutical industries by the development of enzyme/bio-catalyst for large scale production, whereas the Sanger method is used in biological molecules (DNA and RNA) for performing variant studies (27).

A chemical reagent known as phenyl-isothiocyanate is a widely used in HPLC, whereas in the Edman degradation method, the same reagent is also called the Edman reagent (28). In the Edman method, only amino the acid sequence in the peptide can be determined; hence, researchers devised the addition of protein digestion and fractionization protocols to determine the whole sequence of proteins. Protein digestion can be performed with various enzymes, such as proteinase K and fractionization is performed using HPLC, a chromatographic method used to analyse and separate the digested protein samples in liquid form. Later in the 1990s (29), following the discovery of MS, researchers used MS combined with the HPLC technique as an alternative to Edman degradation for the effective identification and for the analysis of protein molecules (30).

The unidentified/unknown proteins from the large sample population can be identified based on two approaches, the bottom-up approach and top-down approach. In the bottom-up approach, the protein is digested into peptides and peptides are separated using MS techniques to determine the protein sequences. The bottom-up approach is also known as shotgun proteomics and HPLC combined with MS are utilized to determine the protein sequences. In the top-down approach, protein is directly separated using MS and other protocols remains the same, as in Shotgun proteomics to determine protein sequences (31,32).

Moreover, recently, proteomic techniques have been explored with several other potential proteins, including heat shock proteins, metabolic enzymes, oxidative proteins, structural proteins, and cell death and signalling regulators that can be utilized in the application of drug or biomarker discovery (33). The applications of proteomics are widely employed by several industry sectors. These include post-translational modification, targeted protein quantification, protein-protein interaction analysis, chemical proteomics, and protein expression profiling (34,35). By contrast, recent developments in proteomics include the monitoring of post-translational modifications by the capillary and microchip electrophoresis techniques (36).

5. *In silico* analysis in proteomics

Various computer-aided platforms have also made analysis easier by performing the majority of the experiment *in silico*. Diverse software and applications have been utilized based on the objective of the study; these make the analysis faster, accurate, as well as time and cost-effective, in comparison to wet-lab experiments. Schrödinger (https://www.schrodinger.com/) is one such widely utilized software for *in silico* experiments and the data can be retrieved using a number of open-source platforms, such as Ensembl (https://asia.ensembl.org/index.html), NCBI (https://www.ncbi.nlm.nih.gov/), EMBL (https://www.embl.org/), Expasy (https://www.expasy.org/resources/uniprotkb-swiss-prot) and DDBJ (https://www.ddbj.nig.ac.jp/index-e.html). Some experiments performed using *in silico* analysis involve variant studies (37), molecular docking (38), critical assessment of structure prediction (CASP) experiments (39), homology modelling (40), pharmacophore modelling, quantitative structure activity relationship (QSAR) modelling (41), *ab initio* methods (42), phylogenetic analysis, sequence similarity searches, primer designing, and computational fluid dynamics (43-45). Over the past decade, bioinformatics has also played a crucial role in computer programming approaches by algorithms (46). Hence as a state of the art, the development of graph algorithms based on protein sequencing and protein identification issues by dynamic programming would be a future goal.

6. Focus on bacterial or fungal cell membrane proteins

Cell membrane proteins and integral components can be studied and are more extensively understood in prokaryotes (bacteria and fungi) when compared to eukaryotic organisms. In bacteria, cell division and synthesis occur with the influence of cell membrane proteins. One such key protein includes, filamenting temperature-sensitive mutant Z (FtsZ) and it is encoded by the FtsZ gene (47). FtsZ is a complex pinpoint in which all cytosolic and membrane proteins are detached. Researchers have previously demonstrated that surfactant-free membrane protein complex separation can be achieved by the presence of FtsZ within penicillin-binding protein (PBP)2/2a nanoparticles by utilizing anti-FtsZ antiserum for the purification of membrane proteins by immuno-affinity chromatography. It was found that FtsZ, PBP2 and PBP2a were captured by styrene-co-maleic acid-lipid particles (SMALP) using an anti-FtsZ antibody, illustrating the ability of the technique to remove significant protein complexes (48).

RodA is a protein belonging to the shape, elongation, division and sporulation (SEDS) family that plays a crucial, yet ambiguous role in cell wall biosynthesis throughout growth, division, and sporulation (49). RodA, a crucial core component of the Rod complex that is highly conserved and serves as a dynamic peptidoglycan-synthesizing tool that mediates the elongation of rod-shaped bacteria. Meeske *et al* (49), performed several biotechnological experiments to prove that SEDS proteins constitute a family of peptidoglycan polymerases and the revelation that SEDS family proteins are peptidoglycan glycosyltransferases (PGTs) with extra cytoplasmic catalytic centres opens an alluring new option to design antibiotics that specifically target this pathway.

Complex regulatory systems ensure that bacteria have the required level of β-barrel outer membrane proteins (OMPs) to facilitate habitat adaptation. The OMP islands are comprised of the Bam complex, which catalyses the insertion of OMPs in the outer membrane, and are distributed throughout the cell (50). The study by Rassam *et al* (51) entrenched a mechanism of binary OMP partitioning by utilizing fluorescent colicins as OMP-specific probes, along with *in vivo* and *in vitro* ensemble and single-molecule fluorescence microscopy, as well as molecular dynamics simulations, to uncover the process underpinning OMP turnover in *Escherichia coli*.

The presence of two adjacent folded subdomains with an IgG-like structure distinguishes the family of proteins known

as the microbial surface components recognising adhesive matrix molecules (MSCRAMMs). The 'dock-lock-latch' mechanism used to bind fibrinogen or the 'collagen hug' mechanism used to bind collagen are two examples of how these promote binding to ligands through procedures that involve significant conformational changes. MSCRAMMs function based on the clumping factors A and B, which play a key role in the pathogenesis of *Staphylococcus aureus* infections (52).

As the incidence of fungal diseases is increasing in developed countries, it is critical to understand the pathological mechanisms of fungi. The stimulation of host defences, including phagocytosis and mediators of humoral immunity, as well as tissue adherence, immune escape mechanisms and host defences are all mediated by cell wall molecules. Endohydrolases, fucosyl transferase, glucuronosyl transferase, chitinases, 1,3-β-glucan synthase, chitin synthase and deacetylase, sialoglycoproteins and uronic acid-containing glycoproteins are some of the enzymes detached to the cell wall and need to be further investigated for understanding the cell mechanisms and functions in fungi (53). To produce antifungal medications against pathogenic fungus including *Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus*, protein kinase-C (PKC) is a viable target. Thus, the growing body of research on enzymes from various species has shown a keen interest in fungal PKCs (54).

7. Proteins in structural biology

As numerous cell-membrane proteins are utilized in therapeutics, it is important to understand the 3D structure of proteins. X-ray crystallography is one such widely used method for the characterization of the 3D structure of proteins. Effective protein extraction, solubilization, stabilization and crystallization are necessary for this method to be successful. For those working to crystallize membrane proteins, each of these processes may provide significant difficulties (55). Pre-crystallization screening needs to be carried out after protein extraction, solubilization and purification to evaluate the stability of proteins. The possibility of crystallization and higher-resolution diffraction improves with the discovery of factors that make pure proteins more stable. A rapid, high-throughput technique utilized to assess the thermostability of solubilized proteins is the thermal denaturation experiment (56).

Dynamic light scattering (DLS) is a rapid and sensitive alternative technique with proven effectiveness in identifying membrane proteins in solution. DLS measures light scattered by the macromolecules in solution, and depending on the rate of light scattering fluctuations, it may assess the size, homogeneity, and stability of samples (57). Based on the protein environment, a precise method of crystallization is applied; this includes vapor diffusion crystallization, *in meso* crystallization and chaperone crystallization (58).

8. Challenges and future perspectives

The electrophoresis protocols are the most commonly used assays for the separation of protein molecules. Among these, SDS-PAGE is generally performed in all cases of separation of biomolecules, whereas in multilevel proteomics, the determination of the peptide, proteoform and protein complex is achieved by capillary electrophoresis utilized with MS (59). In recombinant protein therapeutics to perform quality assessment and unravel protein molecules, capillary isoelectric focusing-MS can be utilized and charge variant analysis can also be performed, demonstrated with bispecific antibody (60). NMR and MS are two techniques widely used to study and determine biological molecules. However, in the field of metabolomics and flux-omics, NMR has certain drawbacks as its resolution, sensitivity and accessibility are low with multiple analytical technical approaches (61). Other than NMR in predicting structure, cryo-electron microscopy also plays a crucial role in predicting the 3D structure of biomacromolecules and these approaches will become advanced in future research applications in analysing the peptide (62). In determining the quality of food components, the majority of food testing laboratories and industries use NMR and MS for quality control; however, to date, there is no NMR database for foodstuffs for comparative analysis and this has yet to be developed (63).

MS methods serve as effective tools for diagnosis and prognosis by differentiating healthy vs. abnormal samples (blood, urine or cerebrospinal fluid). Spatial metabolomics in omics studies enable biomolecule localization, such that it can be utilized with MS for generating imaging MS, and artificial intelligence applications such as deep learning and machine learning will also be utilized as a tool for image analysis (64,65). In the development and advancement of MS in multi-disciplinary studies, the study by Kuo *et al* (66) described five future directions that include constructing a public data repository, creating a future automated platform for usage in a robotic laboratory, moving towards on-site tests, broadening outreach and blindly unravelling biomolecules in routine analysis. Various *in silico* tools and projects based on web developments can also be performed to advance the techniques bioanalytically.

However, beyond all the developed protein technologies (for purification, detection, labelling and fractionization), the major loophole that still exists is the unknown complexity of protein structures in biological cells that can used against the novel therapeutic technology and this can be answered by the development of RNA-sequencing techniques and omics approaches (67). In the recent decade, numerous sequencing techniques for analysing RNA and proteins brought advances to the therapeutic technology against various diseases. Some of the recent ones include Illumina and ion-torrent barcoding technologies (68).

Cell membrane proteins including the protein kinases, GPCR, B-cell lymphoma and chaperones can be utilized in the development of diagnostic and therapeutic products (69-71). Likewise, cell membrane-coated nanoparticles have played a crucial role in nanomedicine, vaccination and targeted drug delivery in various diseases such as cancer, and metabolic disorders (72,73). The various types of bioanalytical analyses used for protein characterization are depicted in Fig. 1, along with a focus on prokaryotic organisms (Table I) for a better understanding of cell membrane complexes and proteins.

9. Conclusion

The present review mainly focused on bacterial and fungal cell membrane proteins, which have been utilized in multiple

Table I. Studies on prokaryotic cell membranes (published over the past 5 years).

Prokaryotic cell membrane proteins	Authors/(Refs.)
Acinetobacter baumannii	Nie *et al* (74)
Pichia pastoris	Chen *et al* (75)
Beauveria bassiana	Ding *et al* (76)
Akkermansia muciniphila	Wang *et al* (77)
Aspergillus flavus	Manju Devi *et al* (78)
Lasiodiplodia theobromae	Peng *et al* (79)
Bacterial outer membrane protein assembly	Nie *et al* (74), Doyle and Bernstein (80), Oluwole *et al* (81), Peterson *et al* (82), Sun *et al* (83), and Tomasek and Kahne (84)
Bacterial respiratory membrane protein complexes	Muras *et al* (85)
Bitopic membrane proteins in bacterial cell division	den Blaauwen and Luirink (86), and Nguyen *et al* (87)
Bacteriophage derived proteins	Grabowski *et al* (88) and Sharma *et al* (89)
Bacterial membrane protein biogenesis	Hegde and Keenan (6), Avila-Calderón *et al* (90), and McDowell *et al* (91)
Fungal membrane protein biogenesis	Diederichs *et al* (92), and Lübeck and Lübeck (93)

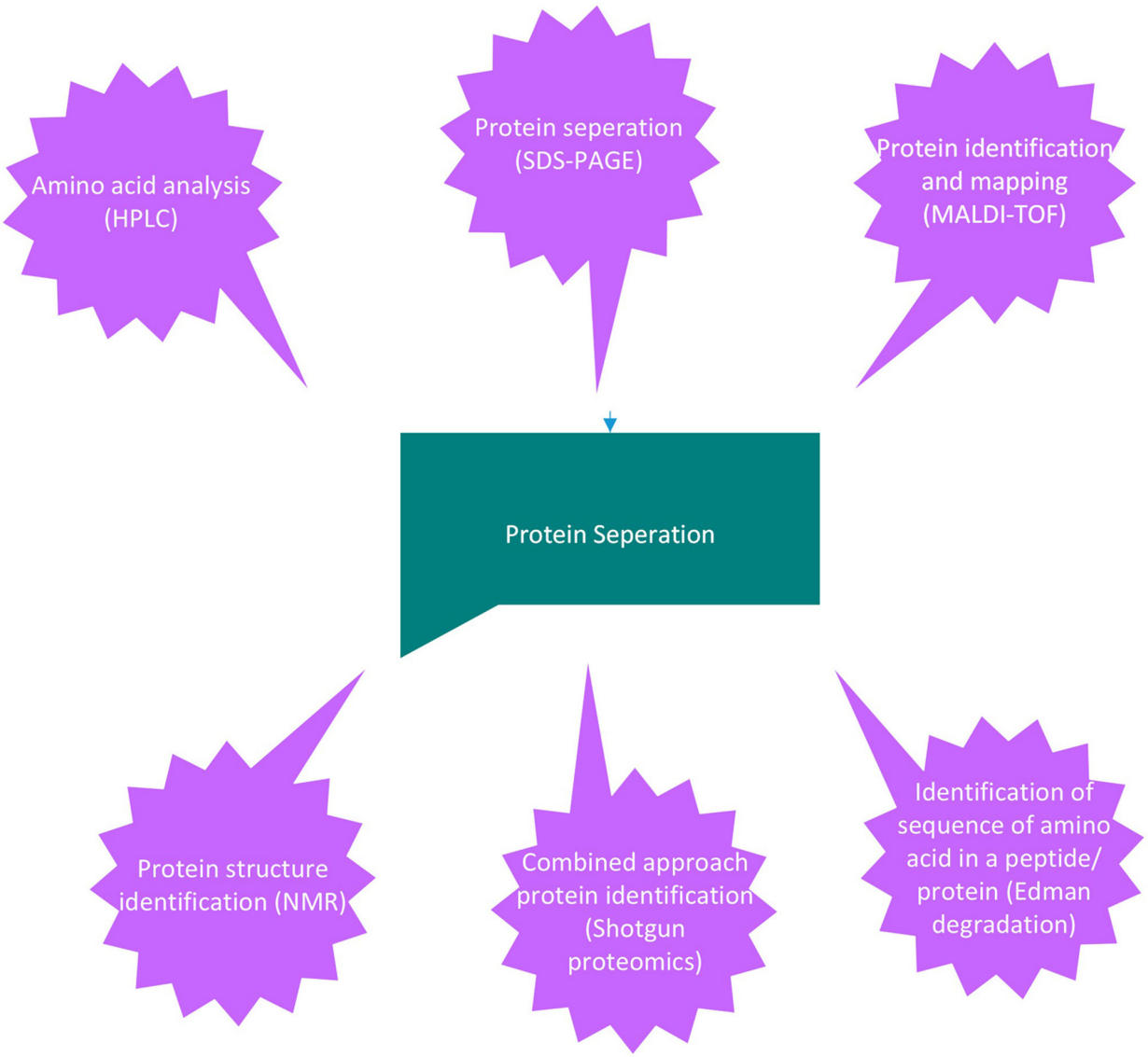

Figure 1. Bioanalytical techniques used for protein characterization. HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; NMR, nuclear magnetic resonance.

aspects of pharmaceuticals to produce therapeutic products. Overall, the bioanalytical techniques, principles behind them, and computer-aided platforms were also discussed in the present study. For researchers who have a very keen interest in proteomics and cell membrane proteins, these studies give the proposal and clarification by helping in the development of problem statements, novelty and applications.

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Authors' contributions

MSAH and IRO were involved in the conceptualisation of the study and in the editing of manuscript. MSAH performed the literature search. KR and IRO reviewed the study. IRO was involved in the submission of final manuscript and in correspondence. All authors have read and approved the final manuscript. Data authentication is not applicable.

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